Characterization of the Fumarase Gene of *Bacillus subtilis* 168 Cloned and Expressed in *Escherichia coli* K12

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The fumarase (*citG*) gene of *Bacillus subtilis* 168 has been identified in a collection of λ phages carrying EcoRI-generated fragments of *B. subtilis* DNA. Regions of the cloned DNA have been subcloned into plasmid vectors, and the ability of prophages and multicopy plasmids to complement *Escherichia coli* and *B. subtilis* fumarase mutations has been examined. Two EcoRI fragments of 1.5 and 5.1 kb are both required for fumarase expression in *E. coli* and *B. subtilis*. The level of fumarase activity from a single copy of the *B. subtilis* *citG* gene expressed in *E. coli* is approximately one-tenth of that from the normal *E. coli* gene; the level is increased by expression from a pBR322-derived multicopy plasmid. The *citG* gene has been located within the cloned DNA by transposon mutagenesis and by expression studies, which have also identified a truncated derivative of this polypeptide as the product of the *citG* gene. The properties of this truncated derivative have indicated the direction of transcription of the *citG* gene.

INTRODUCTION

The *citG* gene of *Bacillus subtilis* 168 encoding the tricarboxylic acid cycle enzyme fumarase (EC 4.2.1.2), is located at 295° on the genetic map (Rutberg & Hoch, 1970; Henner & Hoch, 1980). Lambda phages (λ*citG*) permitting weak complementation of an *Escherichia coli* fumarase-deficient mutant have been isolated from a library of *B. subtilis* DNA cloned in λgtWES (Moir, 1983). The segments of cloned DNA were found to contain two EcoRI fragments of 1.5 and 5.1 kb, which encode a functional *citG* gene, at least part of a spore germination locus (gerA; Moir, 1983), and a genetically defined site of rearrangement in *trpE26* derivatives of *B. subtilis* 168 (Sammons & Anagnostopoulos, 1982). It was also shown that the *citG* mutation of *B. subtilis* could be repaired by transformation with the 5.1 kb fragment purified from agarose gels (Moir, 1983). This paper describes experiments which identify the location of the fumarase gene in the cloned DNA and characterize its expression, transcription polarity and gene product.

METHODS

*Bacterial strains.* The derivatives of *E. coli* K12 and of *B. subtilis* 168 that have been used are listed in Table 1. Strain EJ1535, previously described as 1535 (Guest & Roberts, 1983) and BH2919 (Moir, 1983), is a fumarase-deficient derivative of NK-1.

*Bacteriophages.* Fumarase transducing phages (λ*citG1* and 11) were selected from a gene library provided by Dr J. A. Hoch (Moir, 1983). Other λ*citG* phages, λG142, λG143 and λG144, were isolated in an analogous manner from the same library using λPam3 as helper (this work). All of the λ*citG* phages retained the vector λRS57 marker and only λG143 and λG144 were supF-dependent (i.e. carried at least the λSam7 mutation). Helper phages were essential for transduction by the suppressor-independent phages. Other phages were: λimmK14, a heteroimmune phage that was used for dilsygen construction; λG134, a λfumA phage carrying the *E. coli* fumarase gene (Guest & Roberts, 1983), used for comparative purposes; and λG78N (λnadC aroF genA; Guest & Stephens, 1980), which provided a λRS57 control in post-infection labelling studies.
Plasmids. Plasmids constructed in this study are described in Results and illustrated in Fig. 2. Plasmid pHV33 (Primrose & Ehrlich, 1981) is a hybrid of pBR322 and a staphylococcal plasmid, pC194, that is capable of replication in E. coli and B. subtilis. A derivative of pBR322 carrying the E. coli fumarase (fumA) gene, pG534 (Guest & Roberts, 1983), was used in comparative studies.

Media. The rich medium for subculturing E. coli and for phage propagation was L broth, or LG broth if used for citric acid cycle mutants (Guest, 1981). A peptone medium (Guest, 1981) was used for phage assays and for selective purposes. Media were solidified with agar (Difco) at 10 to 15 g l⁻¹ for plates and 6-5 g l⁻¹ for top layers. Strains of B. subtilis were routinely grown in Difco Penassay Broth and colonies of citG mutants were produced on TBAB agar containing glucose and MnCl₂ (Rutberg & Hoch, 1970). Cit⁻ strains of B. subtilis were selected on minimal agar (Anagnostopoulos & Spizizen, 1961) containing 0-5% sodium DL-lactate as sole carbon source. Accumulation of acids, a characteristic of Cit⁻ strains, was scored on PA agar (Carls & Hanson, 1971).

Antibiotics used for selection after transformation of E. coli by plasmid DNA were ampicillin (at 50 μg ml⁻¹) or tetracycline (at 15 μg ml⁻¹ or 5 μg ml⁻¹; see Results) in L or LG agar. Selection of B. subtilis transformants carrying plasmids was on L agar containing 3 μg chloramphenicol ml⁻¹.

Transformation. Transformation of competent cells of B. subtilis was performed as previously described (Warburg & Moor, 1981). Transformation of E. coli was by the method of Lederberg & Cohen (1974).

Conjugation. The pBR322-derived plasmid pAAM6 was mobilized by and with the F factor of RB308 (pAAM6) by conjugation according to the procedure of Guer (1978).

DNA isolation procedures. Bacteriophage DNA isolation has already been described (Moir, 1983; Spencer & Guest, 1982). Plasmid DNA from E. coli was purified according to Spencer & Guest (1982). pHV33 DNA was prepared from B. subtilis by the method of Niaudet & Ehrlich (1979). Small scale plasmid preparations (Birnboim & Doly, 1979) were used routinely for plasmid screening and transformation.

DNA restriction and ligation. Restriction enzymes EcoRI, HindIII and SalI, and T4 DNA ligase, were purchased from BRL and used under the reaction conditions recommended by the suppliers. DNA fragments were separated by agarose gel electrophoresis and their size estimated as previously described (Spencer & Guest, 1982). Low melting point agarose was obtained from BRL, and phenol extraction of DNA fragments from this agarose was according to the manufacturer’s recommended protocol. Calfe intestinal phosphatase (Boehringer) was purified by the method of Efstratiadis et al. (1977) before use according to the manufacturer’s recommended procedure.

Enzymology. Exponential cultures (250 ml in 2 litre flasks) were grown with shaking in L broth (with ampicillin, 50 μg ml⁻¹, where appropriate) for 4 h at 30 °C (dilysogens) or 37 °C (all other strains) using inocula (5 ml) grown for 16 h in the same medium. Extracts were prepared and assayed for fumarase (EC 4.2.1.2) by measuring the conversion of L-malate to fumarate according to Guest & Roberts (1983) and Hill & Bradshaw (1969). Specific activities are expressed as μmol substrate transformed (mg protein in the high speed supernatant)⁻¹ h⁻¹ at 25 °C.

Analysis of polypeptides formed in UV-irradiated hosts. Polypeptides synthesized after infecting UV-irradiated maltose-grown aerobic cultures of strain S159 (λind) with transducing phages were labelled with L-[35S]methionine as previously described (Guest et al., 1981). Polypeptides expressed from plasmid-borne genes were labelled by the maximell procedure of Sancar et al. (1979) by using glucose-grown cultures of strain AB2480 transformed with the relevant plasmids. Radioactive polypeptides were analysed by SDS-polyacrylamide gel electrophoresis as previously described (Guest & Roberts, 1983).
RESULTS

Properties of λcitG phages

Five λcitG transducing phages have been detected by their ability to complement the fumarase lesion of a fumA mutant of E. coli with the formation of lysogen-filled transduction plaques (see Methods). The gross physiological properties of the phages indicated that they were the products of recombination between the original hybrid phages in the gene bank and the helper phages used to supply integration and other functions needed to facilitate transduction. Restriction maps obtained by digesting the phage DNAs with EcoRI and HindIII, separately and in combination, are shown in Fig. 1. The segments of B. subtilis DNA cloned in these phages were clearly identified as a series of overlapping fragments (6·65 to 10·7 kb) all containing at least two specific EcoRI subfragments of 1·55 and 5·1 kb. It was concluded that the B. subtilis fumarase gene (citG) is very probably expressed from the 6·65 kb segment that is common to all of the λcitG phages. Since the gene bank was originally constructed from a size-selected fraction (10 to 13 kb) of a partial EcoRI digest of B. subtilis DNA, it is likely that the map derived from the cloned fragments corresponds to a continuous 12·4 kb segment of the B. subtilis chromosome, rather than a reassorted set of EcoRI sub-fragments.

Subcloning of DNA into plasmid vectors

To define the location of genes in the cloned segment, DNA fragments from λcitG I and II were subcloned into pHV33, all the plasmid constructions being carried out in E. coli.

Plasmids pAAM3 and pAAM4 (Fig. 2) were constructed by ligation of the complete EcoRI digest of λcitG DNA with EcoRI-digested pHV33 DNA, selecting transformants of ED8654 on L agar containing 15 μg tetracycline ml⁻¹. Plasmids pAAM3, containing the 5·1 kb fragment, and pAAM4, carrying the 1·55 kb fragment, were both identified in a screening of 17 such transformants. Strains carrying pAAM3 grew more slowly on L agar + 15 μg tetracycline ml⁻¹ than those carrying pHV33 or pAAM4, and subsequently tetracycline was used at 5 μg ml⁻¹ for selective purposes.
Fig. 2. Physical maps of plasmids containing segments of the citG region of *B. subtilis* transferred from λcitG to pHV33. Bacterial DNA is denoted by the open bars and vector DNA by stippled bars (pBR322 segment) and hatched bars (pC194 segment). The scale drawings show linear representations of the circular plasmids, their overall sizes (in kb), and the targets for EcoRI (E), HindIII (H), PstI (P) and SalI (S). The approximate positions and polarities of the genes encoding antibiotic resistance are indicated: Cam* (cat); TeP* (let) and Amp* (bla).

In an attempt to obtain plasmids carrying a functional fumarase gene, plasmid DNA from a pool of approximately 600 transformants was used to transform the fumarase mutant EJ1535, selecting transformants on LG + 15 μg tetracycline ml⁻¹. Some 1400 colonies were replica plated to peptone medium containing 15 μg tetracycline ml⁻¹, and three that grew more strongly were screened for plasmid content. Two carried pHV33, and were probably chromosomal revertants to fum⁺, while the third contained a plasmid carrying 1-55 and 5.1 kb fragments; this plasmid was designated pAAM2 (Fig. 2). A second plasmid of identical constitution was obtained from a parallel experiment.

DNA fragments of 7 to 8 kb derived from a partial EcoRI digest of λcitGII were purified by excision of appropriately sized fragments after electrophoresis in a low melting point agarose gel. These were ligated with EcoRI-digested and phosphatase-treated pHV33 DNA. Transformants of ED8654 resistant to 5 μg tetracycline ml⁻¹ were screened and a plasmid carrying 1.55, 5.1 and 0.9 kb fragments was retained and designated pAAM6 (Fig. 2).

A deletion derivative of pAAM2, plasmid pGS96, was constructed by religating pAAM2 after SalI digestion. This plasmid pGS96 retains thebla gene and replication functions of pBR322, the 1.55 kb cloned fragment and the proximal part of the 5.1 kb cloned fragment as far as the single SalI site in the cloned DNA (Fig. 2). The rest of the 5.1 kb fragment, all of pC194, and part of the pBR322 tet gene were lost.

**Complementation and recombination studies in *B. subtilis***

Plasmids pAAM3 and pAAM4 (Fig. 2) were transformed into competent cells of the recombination-proficient strain AM096; only pAAM3 could repair the citG⁴ mutation by recombination. A second genetic marker, gerA1*, was 37% cotransformed with citG⁴*; this linkage is similar to that observed using λcitG phages as donors but is lower than that observed using chromosomal DNA (Moir & Smith, 1983). Complementation of a fumarase defect was tested by transforming pAAM2 and pAAM3 into competent cells of AM099, a recE4 citG⁴ gerA1 strain, selecting chloramphenicol resistance. Transformants were scored for their ability to accumulate acids when grown on PA indicator agar containing chloramphenicol. Strain AM099 carrying pAAM3 formed the characteristic asporogenic and acid-excreting colonies of the Cit− parent; transformants carrying pAAM2 formed mainly Cit⁴ sporogenic colonies, although some Cit− segregants were visible. So, the recombination studies indicate that citG⁴* is carried on the 5.1 kb fragment, and the complementation studies show that the 1.55 kb
Fig. 3. Restriction map of the *B. subtilis* DNA cloned in pGS96 (see Fig. 2). The targets for EcoRI (E), HindIII (H), Clal (C), PstI (P) and SalI (S) are indicated. The positions of Tn1000 insertions studied are indicated by small vertical arrows; those drawn below the map inactivate *citG*, whereas the one drawn above the map does not. The minimum extent of *citG* defined by the Tn1000 insertions is cross-hatched, and the probable extent of *citG* based on the size of the polypeptide product is indicated by the dotted line extensions. The polarity of transcription is indicated by the large arrow.

fragment is also required for expression of a functional fumarase activity in *B. subtilis*. It is therefore concluded that the *citG* gene, or at least the *citG* transcriptional unit, spans the EcoRI target at the junction of 1-55 and 5-1 kb fragments.

**Complementation studies in E. coli**

The plasmids pAAM2, pAAM6 and pGS96 complemented the *E. coli fumA1* mutant, whereas pAAM3 and pAAM4 did not. This confirms that DNA flanking the critical EcoRI target contained in pAAM2, pAAM6 and pGS96 is essential for complementing the fumarase lesion.

**Mapping of the *citG* gene by transposon mutagenesis**

Plasmids such as pBR322 and pHV33 are mobilized by F at a low frequency, the cotransfer usually resulting in their acquiring γδ (Tn1000) from F (Guyer, 1978; De Lencastre et al., 1983). Following its introduction into the F* strain RB308 by transformation, pAAM6 was transferred to EJ1535 in a conjugation experiment. AmpR exconjugants containing pAAM6 were obtained at a frequency of 2 × 10⁻⁴ per donor cell by plating on minimal glucose agar containing 100 µg ampicillin ml⁻¹. Of 177 such exconjugants, screened by replica plating to LG plus tetracycline or chloramphenicol and to peptone agar containing ampicillin, 16 had lost tetracycline resistance, none had lost chloramphenicol resistance, but 33 were now Fum⁻. Plasmids isolated from a number of these Fum⁻ strains were mapped by restriction analysis using EcoRI and SalI, and the map of Tn1000 (Guyer, 1978). The estimated positions of the Tn1000 inserts are shown in Fig. 3. The results indicate that the *citG* gene must extend at least 0-05 kb into the 1-55 kb fragment and at least 0-8 kb into the 5-1 kb fragment. Four Tn1000 inserts which did not inactivate *citG* were mapped on the cloned DNA. They were located within the 5-1 kb EcoRI fragment at 2-3 kb, 2-5–2-6 kb, 2-7 kb and 2-9 kb from its left-hand end as drawn in Fig. 1. Analysis of a sufficient number of inserts to provide narrower maximum limits was not carried out.

**Expression of *B. subtilis*-derived fumarase in E. coli**

*Enzymological studies.* The fumarase activities of derivatives of the *E. coli fumA* mutant carrying the *B. subtilis citG* gene in phage and plasmid vectors were compared with the parental strain, and with analogous derivatives carrying the *E. coli fumA* gene in El34 and pGS54 (Guest & Roberts, 1983).

The dilysogen EJ1535(λimm�34, λG142) contains a single copy of the *citG* gene whereas EJ1535(pAAM2) and EJ1535(pGS96) contain multiple copies. The results, shown in Table 2, indicate that the fumarase activity expressed from a single copy of the *citG* gene is quite significant, but is still 8- to 10-fold lower than that from a single copy of the *fumA* gene (either in the *fumA*⁺ parent or in the mutant carrying a *fumA*⁺ prophage). Expression from pAAM2 and its deletion derivative pGS96 was increased 7-fold and 10-fold respectively over that from a single *citG*⁺ prophage copy. The activities expressed from multiple copies of the heterologous *citG* gene are equivalent to those from single copies of the homologous *fumA* gene. The fumarase
Table 2. Fumarase activities of lysogens and plasmid-containing strains

<table>
<thead>
<tr>
<th>Strain</th>
<th>Relevant genotype</th>
<th>Fumarase specific activity*</th>
</tr>
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<tbody>
<tr>
<td>NK-1</td>
<td>fumA⁺</td>
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</tr>
<tr>
<td>EJ1535</td>
<td>fumA1</td>
<td>&lt;1</td>
</tr>
<tr>
<td>EJ1535(λimm34)</td>
<td>fumA1(λ)</td>
<td>&lt;1</td>
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<tr>
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<td>fumA1(λ, λcitG⁺)</td>
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</tr>
<tr>
<td>EJ1535(λG134)</td>
<td>fumA1(λfumA⁺)</td>
<td>58</td>
</tr>
<tr>
<td>EJ1535(pBR322)</td>
<td>fumA1</td>
<td>&lt;1</td>
</tr>
<tr>
<td>EJ1535(pAAAM2)</td>
<td>fumA1(citG⁺)</td>
<td>46</td>
</tr>
<tr>
<td>EJ1535(pGS96)</td>
<td>fumA1(citG⁺)</td>
<td>65</td>
</tr>
<tr>
<td>EJ1535(pGS54)</td>
<td>fumA1(citA⁺)</td>
<td>308</td>
</tr>
</tbody>
</table>

* Fumarase was assayed as the conversion of malate to fumarate and specific activities are quoted as μmol
  fumarate produced (mg protein)⁻¹ h⁻¹.

activities for fumA⁺ strains in Table 2 are higher than those previously reported (Guest &
Roberts, 1983) and reflect the use of mid-exponential rather than early stationary phase cells.

Identification of the citG product. The post-infection labelling and ‘maxicell’ procedures were
used to detect proteins expressed from phage- and plasmid-cloned genes and to identify the citG
gene product. By using a lysogen as the host in post-infection labelling studies, incorporation of
labelled methionine was limited to a small number of λ proteins and to the products of bacterial
genuses that had been cloned with their own promoters. An autoradiogram of 35S-labelled
polypeptides expressed from three λcitG phages and a control phage (λG78N) revealed only one
product (M, 49000) that is common to all λcitG tracks but not found in the controls (Fig. 4a).
Other products expressed only from λG144 were visible at M, 67000, 25000 and 24000 and they
are presumably encoded by the additional 4-05 kb segment of λG144 (see Fig. 1).

The maxicell procedure was applied to strains containing plasmids that express the citG gene
(pAAAM2 and pGS96), a non-complementing plasmid (pAAAM3) and vector controls (pBR322
and pHV33). These confirmed that a polypeptide of M, 49000 is expressed from the citG region
(Fig. 4b). With pAAAM3, this polypeptide was replaced by one of similar intensity at M, 42000,
potentially a truncated derivative of the M, 49000 product (Fig. 4b).

The identification of the M, 49000 polypeptide as fumarase is strengthened by the properties
of pAAAM3, which would be expected from complementation, recombination and Tn1000
mutagenesis studies to carry part but not all of the citG gene. Furthermore, the replacement of
the M, 49000 polypeptide by one of M, 42000 (whose coding requirement would be about 1-2 kb)
means that the transcription of citG should be initiated within the 5-1 kb EcoRI fragment and
continue outwards into the 1-55 kb fragment, and that the citG coding region could extend about
1-2 kb into the 5-1 kb fragment, as shown in Fig. 3. Labelled polypeptides corresponding to β-
lactamase and chloramphenicol acetyltransferase, at M, 30000 and 26000, respectively, are also
indicated in Fig. 4(b). A number of other specific polypeptides were labelled to a lesser degree in
the maxicells derived from strains carrying pAAAM2, pGS96 and pAAAM3 (Fig. 4b).
However, their significance and origin is as yet uncertain.

DISCUSSION

The fumarase gene (citG) of B. subtilis has been located within a 12-4 kb segment of DNA, by a
combination of biochemical and genetic methods. The coding region has been correlated with a
gene product of M, 49000 which corresponds to about 1-4 kb of DNA, and the direction of
transcription has been established. The location of the citG promoter has not been defined but
the transcriptional unit is not likely to extend beyond the SalI site upstream of the coding region
(Fig. 3), because deletion from this site (to form pGS96) and Tn1000 insertion close to it (Fig. 3)
do not affect citG expression. The nearest genetic marker yet identified, gerA1, maps on the
citG proximal side of a ClaI site (Fig. 3) 1-8 kb from the EcoRI target in citG (I.M.F.,
unpublished data); this mutation does not inactivate fumarase and defines a rather narrower
B. subtilis fumarase gene expression in E. coli

Fig. 4. Autoradiograms of 35S-labelled polypeptides expressed from phage and plasmid genes. (a) Cultures of a UV-irradiated λimm lysogen were labelled for 30 min following infection with ΔcitG (ΔG142-4) and control (ΔG78N) phages. (b) Plasmid-containing strains were labelled for 2 h using the 'maxicell' procedure. Labelled polypeptides were analysed by electrophoresis in SDS-polyacrylamide gels (10%) and the estimated sizes (M_, x 10^-3) of some of the products are indicated. Control cells without phages or plasmids were used for the left-hand tracks in both (a) and (b). The polypeptides corresponding to the B. subtilis fumarase (citG) and a truncated derivative (citGΔ) are indicated. The products of the vector antibiotic resistance genes, and those expressed from E. coli genes of the control (λnadC aroF genA) phage, are shown in parentheses.

limit for the citG unit. The truncated fumarase gene expressed from the 5.1 kb fragment lacks at most the coding capacity for approximately 7000 daltons of the polypeptide, depending on the extent of the short fusion fragment contributed by extension of the reading frame into the pBR322 sequence. This suggests that the citG gene extends for only a few hundred base pairs to the left of the EcoRI target (Fig. 3). This conclusion is consistent with the results from the transposon mutagenesis and is further supported by the properties of a plasmid (pAAM32; I. M. F., unpublished) constructed by insertion of pC194 into the sole HindIII target of pGS96. This plasmid can no longer complement fumA, although a polypeptide indistinguishable in size from the wild-type fumarase is expressed in the maxicell systems. The citG gene therefore extends through the HindIII site, but terminates soon after it. Alterations at the extreme C-terminal portion of the protein appear to critically affect the fumarase activity.
The data presented in this paper provide a basis for current studies aimed at sequencing the fumarase gene for comparison with the analogous E. coli gene. The B. subtilis fumarase is considerably smaller than that of E. coli (M, 61000; Guest & Roberts, 1983; Miles & Guest, 1984) but about the same size as the mammalian enzymes (M, 48500; Beeckmans & Kanarek, 1977). Differences in size between some tricarboxylic acid cycle enzymes have been noted for Gram-positive and Gram-negative organisms, and the former tend to resemble their mammalian counterparts (Weitzman, 1981). The expression of B. subtilis fumarase in E. coli permits only weak complementation, both at the level of growth rate on selective media and in terms of enzyme activity. This may reflect the rate of transcription and translation of the B. subtilis gene in E. coli. Nevertheless, the fumarase protein can be labelled efficiently (Fig. 4) and it may be that the heterologous protein functions less well or is less stable in E. coli. Even when expressed from a multicopy plasmid (Table 2) the activity attained is lower than that from a single copy of the E. coli chromosomal gene.

In addition to citG, the cloned DNA is known to include at least part of a spore germination locus, gerA. Definitive studies of the gerA coding regions are being undertaken. Transposon mutagenesis and complementation studies (A. R. Zuberi, I. M. Feavers and A. Moir, unpublished) have identified at least two intact gerA complementation groups and part of a third within the 5·1 kb fragment; part of one of these complementation groups is present in pGS96. So far, it is not possible to specifically correlate any of these gerA genes with minor labelled polypeptide species expressed in E. coli maxicells.

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REFERENCES


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